In vitro derivation of melanocytes from embryonic neural crest stem cells

Shakhova, Olga ; Sommer, Lukas

Abstract: During development, melanocyte progenitors originate from the neural crest, a transient embryonic structure in vertebrates that gives rise to a variety of cell types including neurons and glia of the peripheral nervous system, smooth muscle cells of the cardiovascular system, chondrocytes and osteoblasts of the craniofacial elements, and pigment cells in the skin. In this chapter, we describe a method for the differentiation of multipotent embryonic neural crest stem cells into differentiated pigmented melanocytes by using in vitro explant culture system. This protocol allows the dissection of genetic and cellular mechanisms regulating neural crest stem cell and melanocyte development. Based on this knowledge it is possible to make predictions about processes that might also be implicated in melanoma initiation and progression.

DOI: https://doi.org/10.1007/7651_2015_305

Title

"*In vitro* derivation of melanocytes from embryonic neural crest stem cells"

Olga Shakhova* and Lukas Sommer

* Corresponding author

Institute of Anatomy, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Abstract

In recent years, it has become apparent that the mechanisms by which cancer cells self-renew, migrate and invade, resemble their embryonic counterparts and that during the steps of the initiation and progression cancer cells can reprogram into embryonic-like cells (1, 2). Melanoma is one of the most aggressive human cancers and it is believed to originate from the genetic deregulation of the cells of the melanocytic lineage. As many other cancers, melanoma cells display the expression of developmental genes and can be reprogrammed when exposed to embryonic environment (3, 4). During development, melanocyte progenitors originate from the neural crest, a transient embryonic structure in vertebrates that gives rise to a variety of cell types including neurons and glia of the peripheral nervous system, smooth muscle cells of the cardiovascular system, chondrocytes and osteoblasts of the craniofacial elements, and pigment cells in the skin. In this chapter, we describe a method for the differentiation of embryonic neural crest stem cells into melanocytes. This protocol allows the dissection of genetic and cellular mechanisms regulating neural crest stem cell and melanocyte development. Based on this knowledge it is possible to make predictions about processes that might also be implicated in melanoma initiation and progression.

Key words: neural crest, stem cells, melanocyte differentiation

1. Introduction

Neural crest cells originate in the ectoderm at the margins of the neural tube and, after a phase of epithelial-mesenchymal transition and extensive migration, settle down in different parts of the body to contribute to the formation of a plethora of different tissues and cell types, including melanocytes (5). Knowledge of neural crest cell biology might help to understand the basic mechanisms underlying growth and metastasis of cancers originating from neural crest derivatives, such as melanoma. Indeed, melanoma-initiating cells have been reported to express the neural crest stem cell marker $p75^{NTR}$/CD271 (low affinity neurotrophin receptor) while lacking expression of markers of differentiated melanocytes, such as tyrosinase, melan-A and melanoma-associated antigen MAGE (6, 7). Moreover, the neural crest stem cell transcription factor Sox10 was recently shown to be expressed in mouse and human melanoma and to promote the formation and maintenance of melanoma (8). Taken together, these recent findings suggest that melanoma cells might utilize genetic and cellular programmes similar to embryonic neural crest stem cells.

After emigration from the neural tube in culture, neural crest stem cells can be isolated and expanded *in vitro* as a monolayer of substrate-attached multipotent cells (9, 10). Moreover, neural crest cells can be propagated *in vitro* at clonal density allowing the investigation of multipotency at the single cell level. Undifferentiated, multipotent neural crest stem cells are characterized by the

expression of the low affinity neurotrophin receptor $p75^{NTR}$/CD271 (9) and the transcription factor Sox10 (11). When grown in a rich medium containing serum or chicken embryo extract, these cells differentiate into a number of neural crest derivatives. Unlike for other neural crest-derived lineages, an instructive growth factor promoting the melanocytic lineage has not yet been identified. However, there are several factors known to promote the survival and proliferation of neural crest-derived melanocytic progenitors, such as stem cell factor (SCF), endothelin-3 (ET-3) and basic fibroblast growth factor (bFGF) (12-16). During development, non-pigmented melanocyte precursors proliferate in the dermis, migrate into the epidermis, and subsequently invade the hair follicle. In the trunk skin of adult mice, the anatomical location of melanocytes is restricted to the hair follicle and therefore, is tightly linked to the regeneration cycle of hair follicles (17, 18). There is accumulating evidence that some human cancers after acquisition of an oncogenetic mutation switch their genetic profile to an embryonic stage (2). Therefore, it is of high interest to identify the genetic programs active in embryonic neural crest stem cells and melanoblasts, adult melanocytic cells, and melanoma cells and to investigate growth factor responsiveness of these cell populations.

In this chapter, we provide a protocol for the differentiation of melanocytes from embryonic multipotent neural crest stem cells. The first step of this protocol consists in the isolation of neural tubes from mouse embryos, followed by plating of the neural tubes onto a fibronectin substrate with subsequent emigration of neural crest stem cells (20 hours). This results in relatively pure population of embryonic neural crest cells (Fig. 1). The second step includes the differentiation of neural crest stem cells into melanocytes by incubation of the explant cultures in a specific melanocyte medium for 10 days *in vitro* (Fig. 1). After 10 days of culture, the majority of neural crest stem cells acquires a melanocytic fate and turns on pigment synthesis, as evident from pigment appearance in the culture dish (Fig. 2). Pigment-positive cells can be easily visualized by inverted microscope using a bright field filter. At this point, cells can be fixed for immunocytochemical analysis of melanocyte-specific markers (Mitf, Dct, Tyr). It is important to note that the addition of fetal calf serum (FCS) is not sufficient for melanocytic differentiation, as only a cocktail of specific growth factors can drive the differentiation process (Fig.3).

This protocol can be applied for the identification of factors regulating proliferation and differentiation of stem cells and melanoblasts and for the generation of cell sources necessary to establish gene expression signatures of embryonic neural crest stem cells producing melanocytes. Comparison of these data with those obtained from adult melanocytes and melanoma cells helps to identify and characterize molecular mechanisms possibly involved in melanoma initiation and progression (8).

2. Materials

1. Hank's Balanced Salt Solution (HBSS) without phenol red (Cat. No. 14025-092, Invitrogen, Life Technologies), Hank's Balanced Salt Solution (HBSS) wihtout Ca^{2+} and Mg^{2+} (Cat. No. 14170-088, Invitrogen, Life Technologies).
2. Phosphate Buffered Saline (PBS) pH 7.4 (1x) (Cat. No. 10010-015, Invitrogen, Life Technologies).
3. Water, Double Processed Tissue Culture Water (Cat. No. W3500, Sigma).
4. Penicillin and Streptomycin (Cat. No. 15140-122, Invitrogen, Life Technologies).
5. Sterile cell culture plastic pipettes individually wrapped (2, 5, 10, 25 ml).
6. Plasticware: cell culture dish (35 mm x 10 mm) (Corning Incorporated, Corning. Cat. No. 430165).
7. Dispase I (neutral protease, grade I), 10x approx. 2 mg (Cat. No. 04 942 086 001, Roche).
8. Substrate coating: 0.1 % solution of fibronectin (Cat. No. F1141, Sigma).
9. Syringe Driver Filter Unit, 0.22 μm (Cat. No. SLGPO33RS, Millex-GP).
10. 50 ml of neural crest medium is composed of 47.1 ml of $L15CO_2$, 50 mg BSA (1mg/ml), 2 ml of 1:1:2, 50 μl insulin (5 μg/ml), 100 μl putrescine (16 μg/ml), 10 μl progesterone (20 nM), 15 μl selenious acid (30 nM), 0.5 μl dexamethasone, 143 μl glycerol, 50 μl vitamin E (5 μg/ml), 500 μl Mix 7, 20 μl bFGF (10 ng/ml) and 100 μl transferrin (100 μg/ml).
11. To prepare $L15CO_2$, mix 3.675 g of Leibovitz's L-15 powder (Cat. No. 41300-021, Invitrogen, Life Technologies), 0.019 g imidazole (Cat. No. I-0250, Sigma), 1.6 ml of stable vitamin mix and 288 ml of water. Adjust pH to 7.4. Separately, mix 0.8 g sodium bicarbonate (Cat. No. S-5761, Sigma), 120 μl phenol red, 59 ml of water and apply CO_2 until the solution turns yellow. Add sodium bicarbonate solution and repeat CO_2 step. Store at 4^0 C.
12. To prepare stable vitamin mix, mix 0.6 g aspartic acid (Cat. No. A-4534, Sigma), 0.6 g L-glutamic acid (Cat. No. G-8415, Sigma), 0.6 g L-proline (Cat. No. P-4655, Sigma), 0.6 g L-cysteine (Cat. No. C-7602, Sigma), 0.2 g p-aminobenzoic acid (Cat. No. 42,976-7, Aldrich), 0.2 g 3-aminoproprionic acid (Cat. No. A-9920, Sigma), 80 mg vitamin B_{12} (Cat. No. V-6629, Sigma), 0.4 g myo-inositol (Cat. No. I-7508, Sigma), 0.4 g choline chloride (Cat. No. C-7527, Sigma), 1 g fumaric acid (Cat. No. F-8509, Sigma), 16 mg coenzyme A (Cat. No. C-4282, Sigma) (solution 1). Separately, dissolve 0.4 mg D-biotin (Cat. No. B-4639, Sigma) and 100 mg DL-α-lipoic acid (Cat. No. T-1395, Sigma) in 10 ml of water (solution 2). Add 2 ml of solution 2 to solution 1, mix and prepare 1.5 ml aliquots.
13. To prepare 1:1:2, dissolve 60 g dextrose (Cat. No. G-7021, Sigma) in 160 ml of water (adjust the volume to 200 ml after dextrose is dissolved). Add 100 ml glutamine (Cat. No. G-6392, Sigma) (200 mM)

and 100 ml of penicillin-streptomycin solution. Prepare 2 ml aliquots and store at -20^0 C.

14. To prepare Mix7, dissolve 630 mg DL-ß-hydroxybutyrate (Cat. No. H-6501, Sigma) in 10 ml water. Dissolve cobalt chloride (Cat. No. C-8661, Sigma) in water (10 mg/ml) and subsequently prepare 25 µg/ml solution by diluting it in L15CO_2. Dissolve biotin in DMSO (10 mg/ml) and dilute it in L15CO_2 (1mg/ml). Dissolve oleic acid (Cat. No. O-7501, Sigma) in water (2.8 mg/ml) and subsequently in L15CO_2 up to 10 µg/ml. Dissolve prostaglandin (Cat. No. P-5515, Sigma) in 95% ethanol to obtain 1 mg/ml solution and dilute in L15CO_2 (10 µg/ml). Dissolve 3,3',5-triodo L-threonine (Cat. No. T-6397, Sigma) in DMSO (up to 10 mg/ml) and dilute it further in L15CO_2 to 67.5 µg/ml. Mix 5 ml of each solution with 15 ml of L15CO_2, prepare 550 µl aliquots and store at -20^o C.
15. Melanocyte differentiation media is composed of DMEM/F12 GlutaMAX Dulbeccos's Modified Eagle Medium (Cat. No. 31331-028, Gibco by Life Technologies) supplemented with penicillin-streptomycin (P/S), L-Glutamine (L-Gln) 200 mM (Cat. No. 25030-024, Invitrogen, Life Technologies) and 5 % FCS. To prepare 160 µM stock solution of TPA, dilute TPA (12-*O*-tetradecanoyl phorbol-13-acetate, Cat. No. P1585, Sigma) into ethanol and store it at -20^O C (final concentration is 160 nM). mSCF (stem cell factor/mast cell growth factor, mouse, recombinant, Cat. No. 250-03, Peprotech). Prepare 50µg/ml stock solution by dissolving in PBS (final concentration is 50 ng/ml). ET-3 (endothelin 3, human, Cat. No. E9137, Sigma). Prepare 50 µM stock solution by dissolving in PBS (final concentration is 100 nM). bFGF (fibroblast growth factor-basic, human, recombinant, Cat. No. 100-18B, PeproTech). Prepare 25 µg/ml stock solution by dissolving in PBS (final concentration is 25 ng/ml). α-MSH (α-melanocyte stimulating hormone, synthetic, Cat. No. M4135, Sigma). Prepare 100 µg/ml stock solution by dissolving in water (final concentration is 100 ng/ml). Dex (dexamethasone, Cat. No. D4902, Sigma). Prepare 1mM stock by dissolving in 95% ethanol (final concentration is 1 nM).

3. Methods

1. Isolate E9.5 embryos from the uterus of time-mated females and place them into 10-cm petri dish with sterile HBSS without phenol red. From this step onwards, embryos and tissue should be stored on ice.
2. Dissect the trunks and place them into a new 3.5 cm dish containing HBSS without phenol red. Prepare a mixture for enzymatic digestion. For this, dissolve 1 vial of Dispase I into 12 ml of HBSS (without Ca^{2+} and Mg^{2+}). Add 12 ml of dissolved Dispase I

into 6 well plate (2 ml per each well) and transfer the trunks into this mixture for 5 min at 4^0 C using a Pasteur pipette.

3. In order to isolate neural tubes, carefully triturate the trunks using a Pasteur pipette. After the trituration step, transfer neural tubes into DMEM media supplemented with 10% FCS to stop the enzymatic digestion step (at this step one can collect all neural tubes again in one 3.5 cm petri dish).
4. Prepare 3.5 cm petri dishes for culturing: coat every 3.5 cm (Corning petri dish) with fibronectin. Add 1 ml of culture media into 3.5 cm dishes and place tissue culture dishes into the cell culture incubator.
5. Plating of neural tubes: remove media (by leaving around 200 μl) and place carefully neural tube on the bottom of 3.5 cm plate (we usually plate 2-3 neural tubes per dish). This step has to be done under the dissecting microscope. Try to avoid producing air bubbles as in this case, neural tubes will not attach. Use 20 μl Gilson pipette at this step.
6. After 30 minutes of incubation at 37^0 C in the CO_2 incubator, carefully add the remaining media (removed at step 5 and collected into 50 ml tube) by controlling whether neural tubes are still attached.
7. Incubate the neural crest explants at 37^0 C with 5% humidified CO_2 for 20 hours to allow neural crest cells to emigrate from neural tube onto the plastic dish. Examine neural crest explants by phase microscopy.
8. To initiate melanocyte differentiation, remove neural crest medium and add melanocyte medium (see components above).
9. Medium should be changed every 3 days until appearance of visible pigmented cells.

4. Notes

1. The developmental stage of the embryos is very important (if you start the preparation using embryos older than E9.5, the yield of neural crest stem cells might be very low).
2. Be careful when coating 3.5 cm dishes with fibronectin solution, do not let it dry.
3. As the trituration step is critical, it is recommended to incubate the trunks at 4^0 C exactly for 5 minutes and immediately proceed with the trituration.
4. For the melanocytic differentiation step, add the melanocyte medium as soon as neural crest stem cells have emigrated. Delay might result in the acquisition of non-melanocytic fates due to spontaneous differentiation.

References:

1. Palmer, N. P., Schmid, P. R., Berger, B., and Kohane, I. S. (2012) A gene expression profile of stem cell pliripotentiality and differentiation is conserved across diverse solid and hematopoietic cancers, *Genome Biol* 13, R71.
2. Youssef, K. K., Lapouge, G., Bouvree, K., Rorive, S., Brohee, S., Appelstein, O., Larsimont, J. C., Sukumaran, V., Van de Sande, B., Pucci, D., Dekoninck, S., Berthe, J. V., Aerts, S., Salmon, I., Del Marmol, V., and Blanpain, C. (2012) Adult interfollicular tumour-initiating cells are reprogrammed into an embryonic hair follicle progenitor-like fate during basal cell carcinoma initiation, *Nat Cell Biol* 14, 1282-94.
3. Yu, L., Harms, P. W., Pouryazdanparast, P., Kim, D. S., Ma, L., and Fullen, D. R. (2010) Expression of the embryonic morphogen Nodal in cutaneous melanocytic lesions, *Mod Pathol* 23, 1209-14.
4. Strizzi, L., Hardy, K. M., Kirsammer, G. T., Gerami, P., and Hendrix, M. J. (2011) Embryonic signaling in melanoma: potential for diagnosis and therapy, *Lab Invest* 91, 819-24.
5. Shakhova, O., and Sommer, L. (2008-2010) Neural crest-derived stem cells, *Stem Book*, Cambridge (MA): Harvard Stem Cell Institute, May 4.
6. Boiko, A. D., Razorenova, O. V., van de Rijn, M., Swetter, S. M., Johnson, D. L., Ly, D. P., Butler, P. D., Yang, G. P., Joshua, B., Kaplan, M. J., Longaker, M. T., and Weissman, I. L. (2010) Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271, *Nature* 466, 133-7.
7. Civenni, G., Walter, A., Kobert, N., Mihic-Probst, D., Zipser, M., Belloni, B., Seifert, B., Moch, H., Dummer, R., van den Broek, M., and Sommer, L. (2011) Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth, *Cancer Res* 71, 3098-109.
8. Shakhova, O., Zingg, D., Schaefer, S. M., Hari, L., Civenni, G., Blunschi, J., Claudinot, S., Okoniewski, M., Beermann, F., Mihic-Probst, D., Moch, H., Wegner, M., Dummer, R., Barrandon, Y., Cinelli, P., and Sommer, L. (2012) Sox10 promotes the formation and maintenance of giant congenital naevi and melanoma, *Nat Cell Biol* 14, 882-90.
9. Stemple, D. L., and Anderson, D. J. (1992) Isolation of a stem cell for neurons and glia from the mammalian neural crest, *Cell* 71, 973-85.
10. Kleber, M., Lee, H. Y., Wurdak, H., Buchstaller, J., Riccomagno, M. M., Ittner, L. M., Suter, U., Epstein, D. J., and Sommer, L. (2005) Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling, *J Cell Biol* 169, 309-20.
11. Paratore, C., Goerich, D. E., Suter, U., Wegner, M., and Sommer, L. (2001) Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling, *Development* 128, 3949-61.
12. Langtimm-Sedlak, C. J., Schroeder, B., Saskowski, J. L., Carnahan, J. F., and Sieber-Blum, M. (1996) Multiple actions of stem cell factor in neural crest differentiation in vitro, *Dev Biol* 174, 345-59.

13. Sieber-Blum, M. (1998) Growth factor synergism and antagonism in early neural crest development, *Biochem Cell Biol* 76, 1039-50.
14. Lahav, R., Dupin, E., Lecoin, L., Glavieux, C., Champeval, D., Ziller, C., and Le Douarin, N. M. (1998) Endothelin 3 selectively promotes survival and proliferation of neural crest-derived glial and melanocytic precursors in vitro, *Proc Natl Acad Sci U S A* 95, 14214-9.
15. Le Douarin, N. M., Creuzet, S., Couly, G., and Dupin, E. (2004) Neural crest cell plasticity and its limits, *Development* 131, 4637-50.
16. Sommer, L. (2011) Generation of melanocytes from neural crest cells, *Pigment Cell Melanoma Res* 24, 411-21.
17. Osawa, M. (2008-2009) Melanocyte stem cells, *Stem Book*, Cambridge (MA): Harvard Stem Cell Institute, June 30.
18. Blanpain, C., and Fuchs, E. (2006) Epidermal stem cells of the skin, *Annu Rev Cell Dev Biol* 22, 339-73.

Figure 1. **A shematic representation of a protocol of *in vitro* derivation of melanocytes from embryonic neural crest stem cells.** In the first step, a neural tube is isolated from the mouse embryo at the embryonic day 9.5 and plated onto the culture dish, previously coated with fibronectin. After 20 hours of incubation, a population of undifferentated neural crest stem cells emigrate from the neural tube onto fibronectin-coated surface. In the second step of this protocol, neural crest stem cells can be stimulated to acquire melanocytic differentiation by the addition of culture media containing specific growth factors. NT, neural tube. ET-3, endothelin-3. mSCF, mouse stem cell factor. α-MSH, alpha-melanocyte-stimulating hormone. TPA, 12-O-tetradecanoylphorbol-13-acetate. Dex, dexamethasone. FGF2, basic fibroblast growth factor.

Figure 2. **An example of melanocytes derived from embryonic neural crest stem cells.** After 10 days of culture (media has to be refreshed every 2-3 days) in melanocyte media supplemented with specific growth factors, one can observe an increasing number of pigmented cells. These cells can be easily visualized using bright field microscope.

Figure 3. **Requirement of specific growth factors for the melanocytic differentation.** Only the specific growth factors (ET-3, mSCF, α-MSH, TPA, Dex, FGF2) can support the acquisition and survival of the melanocytic differentiation. FCS alone is not sufficient to drive the melanocytic differentation from the embryonic neural crest stem cells. FCS, fetal calf serum.

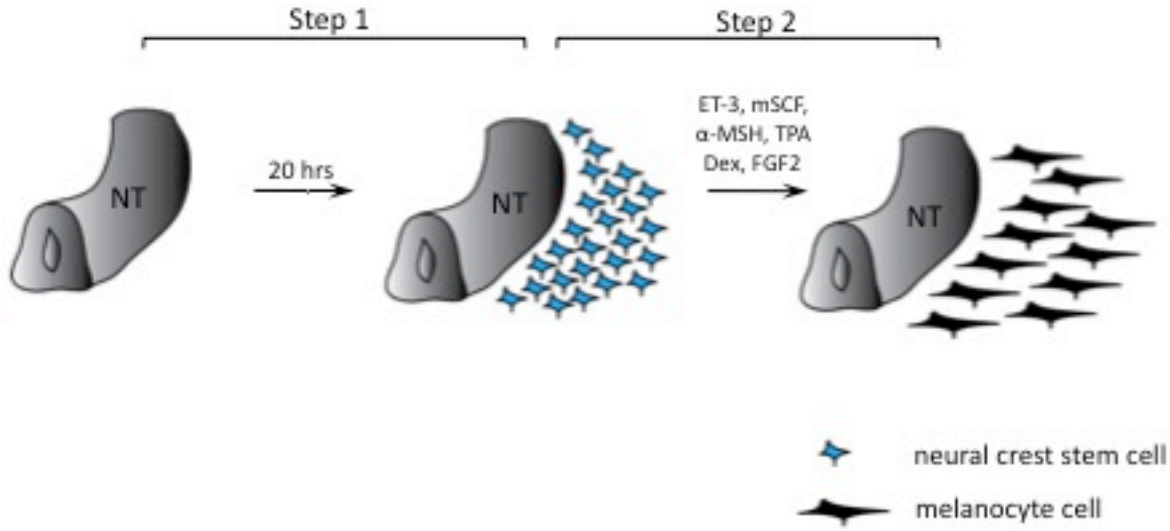
Step 1
Step 2
NT
20 hrs
NT
ET-3, mSCF,
α-MSH, TPA
Dex, FGF2
NT
neural crest stem cell
melanocyte cell

Figure 1.

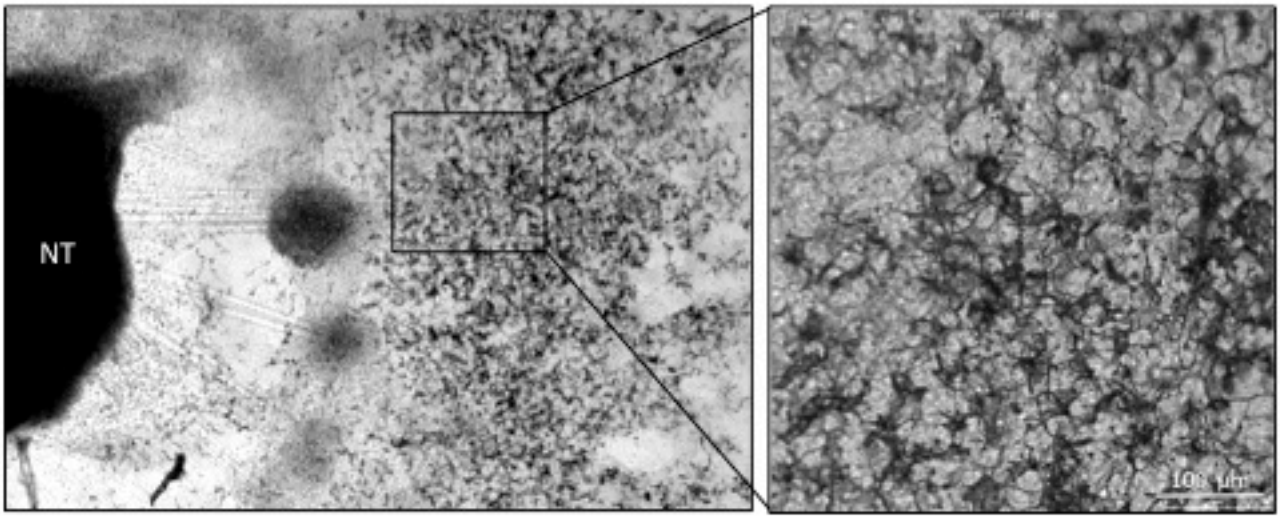

Figure 2.

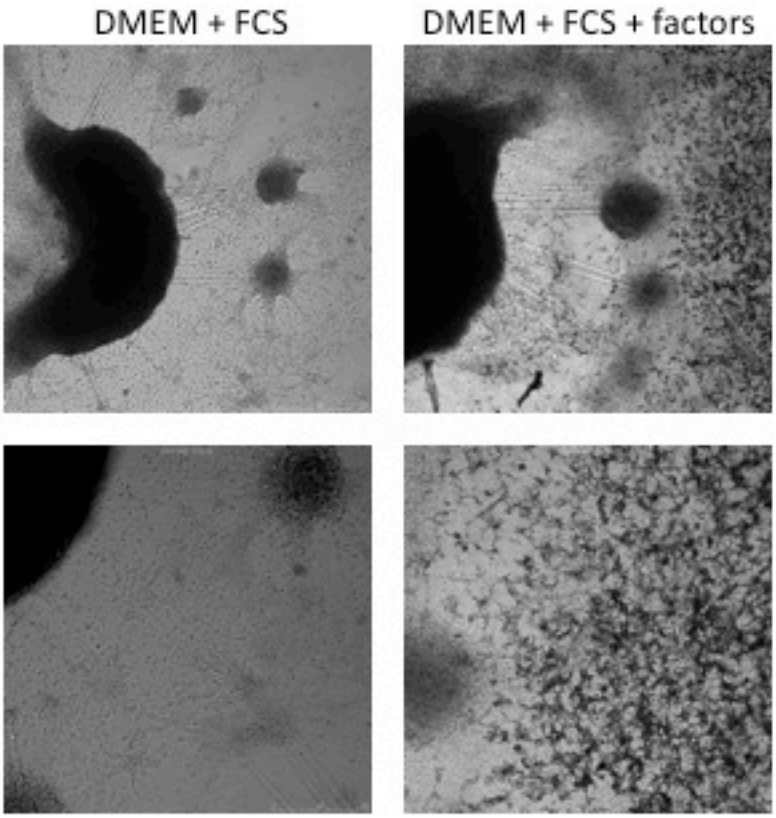

Figure 3.